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USC-HN2, A NEW MODEL CELL LINE FOR RECURRENT ORAL CAVITY SQUAMOUS CELL CARCINOMA WITH IMMUNOSUPPRESSIVE CHARACTERISTICS

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Abstract

Objectives—Head and neck squamous cell carcinomas (HNSCC) are common and aggressive tumors that have not seen an improvement in survival rates in decades. These tumors are believed to evade the immune system through a variety of mechanisms and are therefore highly immune modulatory. In order to elucidate their interaction with the immune system and develop new therapies targeting immune escape, new pre-clinical models are needed.

Materials and Methods—A novel human cell line, USC-HN2, was established from a patient biopsy specimen of invasive, recurrent buccal HNSCC and characterized by morphology, heterotransplantation, cytogenetics, phenotype, gene expression and immune modulation studies and compared to a similar HNSCC cell line; SCCL-MT1.

Results and Conclusion—Characterization studies confirmed the HNSCC origin of USC-HN2 and demonstrated a phenotype similar to the original tumor and typical of aggressive oral cavity HNSCC (EGFR⁺CD44v6⁺FABP5⁺Keratin⁺ and HPV⁻). Gene and protein expression studies revealed USC-HN2 to have highly immune-modulatory cytokine production (IL-1 β , IL-6, IL-8, GM-CSF, and VEGF) and strong regulatory T and myeloid derived suppressor cell (MDSC) induction capacity in vitro. Of note, both USC-HN2 and SCCL-MT1 were found to have a more

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robust cytokine profile and MDSC induction capacity when compared to 7 previously established HNSCC cell lines. Additionally, microarray gene expression profiling of both cell lines demonstrate up-regulation of antigen presenting genes. Because USC-HN2 is therefore highly immunogenic, it also induces strong immune suppression to evade immunologic destruction. Based upon these results, both cell lines provide an excellent model for the development of new suppressor cell-targeted immunotherapies.

Keywords

Head and Neck Squamous Cell Carcinoma; cell line; tumor immune tolerance; Myeloid Derived Suppressor Cells (MDSC); Human papillomavirus (HPV)

INTRODUCTION

Head and neck cancer is the sixth most common solid tumor malignancy worldwide, and despite available surgical and adjuvant therapies, continues to cause significant morbidity and mortality^{1,2}. These predominantly (>90%) squamous cell cancers can arise from the epithelium of the sinonasal tract, oral cavity, pharynx, or larynx, and are associated with a history of tobacco smoking, excessive alcohol consumption, and human papillomavirus (HPV) infection^{1,3-7}. The five-year survival rate for patients with head and neck squamous cell carcinoma (HNSCC) is poor (30–40%) and has shown only marginal improvement in the past four decades, highlighting the need for new therapeutic approaches^{3,8}. The immunologic properties of HNSCC are of particular interest in this new era of cancer immunotherapy⁹. It is now recognized that the immune system is capable of recognizing and eliminating cancer cells in the host, but that tumors adapt to evade and escape immune attack¹⁰. Numerous groups have provided evidence of the immunomodulatory effects of HNSCC, including the local and regional suppression of the immune system by interleukins (IL-6, IL-10), vascular endothelial growth factor (VEGF), cyclo-oxygenase 2 (COX2), and matrix metalloproteinases¹¹⁻¹⁵. Specifically, individuals with aggressive HNSCC tumors are observed to have a Th2-shifted immune response and decreased cell-mediated (Th1) immunity^{11,16,17}. Immunotherapy is a promising modality for the treatment of HNSCC because it is targeted, systemic, and generates immunological memory that can prevent recurrent disease¹⁰.

Cancer cell lines are important models for pre-clinical studies of disease progression and the development of new therapies. Few HNSCC cell lines are publicly available for such studies [12 HNSCC cell lines currently available through the American Tissue-type Cell Collection (ATCC)], and many lack complete characterization, particularly with respect to immune-modulatory characteristics. We describe the establishment and characterization of a unique HNSCC cell line, USC-HN2, derived from an invasive, recurrent buccal squamous cell carcinoma tumor. Additionally, USC-HN2 was compared to a previously established HNSCC cell line, SCCL-MT1, which has not been characterized in the literature and was also found to have strong immune-modulatory activity, a pre-requisite for tumor models that can facilitate the development of new immunotherapies for these cancers.

MATERIALS AND METHODS

Cell lines and tissues

Tumor cell lines were obtained from ATCC or gifted to the Epstein laboratory and authenticity was verified by cytogenetics and surface marker analysis as described previously¹⁸. HNSCC tumor biopsy samples were obtained and used under USC Keck School of Medicine IRB-approved protocol HS-09-00048.

Establishment of cell line USC-HN2

Tumor explants were used to develop the USC-HN2 cell line, as described previously¹⁸. After establishment of the cell line, interval screening was performed using MycoAlert Mycoplasma Detection Kit (Lonza, Rockland, ME). Cell doubling time was determined for USC-HN2 by cell count measurements at 24 hour intervals for one week.

Heterotransplantation in Nude mice

Eight-week-old female Nude mice (n=3, Simonsen Laboratory, Gilroy, CA) were injected with cultured USC-HN2 cells for heterotopic (s.c. flank, 7.5×10^6 cells) or orthotopic (base of the tongue, 3×10^6 cells) heterotransplantation studies. Tumor measurements were made twice weekly and animals were sacrificed two (oral cavity) or four (flank) weeks after implantation. Institutional Animal Care and Use Committee-approved protocols were followed.

Immunohistochemistry (IHC)

Cytospin preparations of USC-HN2 cells from culture and tissue sections of the patient biopsy and heterotransplanted tumors were used for IHC studies, as described previously^{18,19}. Wright-Giemsa staining (Protocol Hema 3, Fisher, Kalamazoo, MI) of USC-HN2 and SCCL-MT1 cytospin preparations was performed to assess and compare morphology, as described previously^{18,19}. Both USC-HN2 cytospin and paraffin tissue slides were stained for specific antigens with monoclonal antibodies including CD44 (DF1485; Dako Corp., Carpinteria, CA), E-cadherin (4A2C7; Invitrogen, Carlsbad, CA), EGFR (E30; Biogenex, San Ramon, CA), keratin (AE1/AE-3; Covance, Berkeley, CA), p53 (1801; CalBiochem, San Diego, CA), Rb (RbG3-245; BD Biosciences, San Diego, CA), p16 (INK4), and FABP5 (311215) (R&D Systems, Minneapolis, MN). Observation, evaluation, and image acquisition were made as described previously^{18,19}.

Analysis of surface markers by flow cytometry

Single cell suspensions (10^6 cells in 100 μ l) in 2% FCS in PBS were stained with fluorescence-conjugated antibodies as described previously^{18,19}. For intracellular stains, buffer fixation/permeabilization (eBioscience, San Diego, CA) was performed prior to staining. Antibodies were purchased from BD Biosciences: CD24 (ML5), CD74 (M-B741), E-cadherin (36/Ecadherin), EGFR (EGFR1), Nanog (N31-355), Oct 3/4 (40/Oct-3), SOX2 (245610), and isotype controls; Santa Cruz Biotechnology (Santa Cruz, CA): IL-13R α 2 (B-D13), and c-kit (104D2); Abcam (Cambridge, MA): CD44v6 (VFF-7); and eBioscience: CD133 (TMP4) and isotype controls.

Cytogenetics and *in situ* hybridization

Karyotype analysis using Giemsa staining and *in situ* hybridization for HPV DNA sequences were performed by the Division of Anatomic Pathology, City of Hope Medical Center (Duarte, CA) using early passages of USC-HN2 and SCCL-MT1. Single color FISH for HPV was performed using Enzo Life Sciences HPV16/18 probe (ENZO-32886, Plymouth Meeting, PA) followed by tyramide signal amplification (TSA kit#21, Invitrogen). Multi-color FISH using probes for unique chromosomal abnormalities found in USC-HN2 (Abbott MYC breakapart probe 8q24 and Abbott probe 5-9-15) confirmed the origin of the cell line from the patient tumor biopsy.

Microarray gene expression profiling

Total RNA was isolated from USC-HN2 and SCCL-MT1 using RNeasy Mini Kit (Qiagen, Valencia, CA) and analyzed by microarray, as previously described¹⁸. Human universal RNA (huRNA; Stratagene, Santa Clara, CA) was used as a common reference for all

experiments. For data analysis, data files were uploaded into mAdb database and analyzed by the software tools provided by the Center for Information Technology (CIT), NIH. SAM (Significance Analysis of Microarray) and *t*-test analyses were performed to identify differentially expressed genes. In addition, GSEA (Gene Set Enrichment Analysis)²⁰ provided in mAdb was also performed to distinguish groups of differentially expressed genes in these cell lines.

TP53 mutation analysis

Genomic DNA isolated as above was amplified using primers for exons 5–9 of *TP53*, as described by Dai *et al*²¹. Purified PCR products were sequenced by the USC DNA core facility using ABI 3730 DNA Analyzer (Applied Biosystems) and screened for mutations using BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Cytokine and oncogene analysis by quantitative(q)RT-PCR

Gene expression analyses by qRT-PCR were performed on USC-HN2 and SCCL-MT1 cell lines as described previously¹⁸.

Measurement of tumor-derived factors by ELISA

Three-day supernatants were collected from cell line cultures at 90% confluence, 0.2µm-filtered to remove cell debris, and analyzed for protein levels of IL-1β, IL-6, IL-8, TNFα, VEGF, and GM-CSF using ELISA DuoSet kits (R&D). Plate absorbance was read on an ELX-800 plate reader (Bio-Tek, Winooski, VT) and analyzed using KC Junior software (Bio-Tek).

Induction of regulatory T cells and myeloid-derived suppressor cells

USC-HN2 and SCCL-MT1 cell lines were tested for induction of regulatory T cells (Treg) and myeloid-derived suppressor cells (MDSC) as described previously^{22,23}. Briefly, PBMCs obtained from healthy volunteers (under USC Keck School of Medicine IRB-approved protocol HS-06-00579) were co-cultured in complete medium with tumor cell lines for one week. After co-culture, CD33⁺ or CD4⁺CD25^{high} cells were isolated by magnetic bead separation and tested for suppressive function by their ability to inhibit the proliferation of fresh, autologous CD3/CD28-stimulated CFSE-labeled (3µM) T cells *in vitro*. T cell proliferation was measured by flow cytometry after three days.

Statistical analysis

To identify statistically significant differences in gene and protein expression by HNSCC cell lines and T cell proliferation, one-way ANOVA followed by Dunnett post-test was applied. Statistical analyses for microarray experiments are described above. Statistical tests were performed using GraphPad Prism software (La Jolla, CA) at a significance level of $\alpha=0.05$. Graphs and figures were produced using GraphPad Prism, Microsoft Excel, and Adobe Illustrator and Photoshop software.

RESULTS

Case report of patient with recurrent invasive left buccal squamous cell carcinoma

The patient is an 81-year-old female with a 50-pack-year history of tobacco smoking and occasional alcohol consumption and a past medical history of recurrent left sided oral cancer. The patient was initially diagnosed in April, 2002 following surgical resection of a moderate-to-poorly differentiated SCC of the oral cavity with a second surgical resection for recurrence in August, 2002. The patient underwent a third surgical resection for suspected recurrence in August, 2009 which revealed a 4cm moderately differentiated SCC of the

buccal mucosa with bone and perineural invasion, but no evidence of vascular invasion or tumor metastasis to submental, submandibular, maxillary, oral cavity, or floor of mouth lymph nodes (Stage IV, T4N0M0; Figure 1A). The patient did not receive any radiation or chemotherapy treatment and is currently tumor-free and continues to have routine follow-up at the USC University Hospital.

Establishment of USC-HN2 cell line

The USC-HN2 cell line was derived from the patient's recurrent buccal mucosal SCC resected in August, 2009 using culture flask-adherent explant fragments. After 2–3 weeks, tumor cells were removed by trypsinization and placed in petri dishes for cloning procedures required to isolate a cell line from normal stromal cells. USC-HN2 cells have rapid doubling time of 22 hours, which is comparable to the previously reported growth rates of other HNSCC cell lines (26.5 hours)⁸. Once a morphologically uniform population of cells was established, several freezings were performed to obtain early passages of USC-HN2 and several vials were sent to ATCC for distribution to other investigators.

Heterotransplantation in Nude mice

USC-HN2 cells from cell culture were injected in the oral cavity or subcutaneously in athymic Nude mice (n=3) and tumors were excised after two (tongue) or four (subcutaneous) weeks (Figure 1A). Subcutaneous tumors grew to between 110mm³ and 150mm³ and oral cavity tumors were excised once visible tumors had grown (3mm³; data not shown). H&E stained sections of the heterotransplants showed a moderately to poorly differentiated, keratinizing SCC. Surrounding the invasive tumor, a mild to moderate chronic and acute inflammatory infiltrate was present. These findings demonstrate that USC-HN2 is transplantable in xenograft models and that heterotransplanted tumors closely resembled the original tumor.

Morphology of USC-HN2 cell line is typical of oral cavity squamous cell carcinoma

Phase-contrast photomicrographs of cultured cells and Wright-Giemsa stained cytopspins were used to assess the morphology of USC-HN2 cell line as compared to the established HNSCC cell line SCCL-MT1 (Figure 1B). Both cell lines demonstrated characteristic features of oral cavity squamous cell carcinoma. USC-HN2 cells showed nuclear pleomorphisms with prominent nucleoli, frequent mitotic figures, and an abundant, vacuolated cytoplasm.

Cytogenetics

Cytogenetic analysis of USC-HN2 was performed in order to confirm the unique identity of this cell line and origin from the original tumor sample. All mitotic cells collected for GTG-band analysis from USC-HN2 cell cultures were clonally abnormal. The karyotype of USC-HN2 contains characteristic features of HNSCC, including isochromosome formation with resultant loss/deletion of the short arm of chromosome 8, and breakpoints at or near the centromeres (Figure 2A)¹. Multi-color FISH shows similar chromosomal abnormalities in the original tumor biopsy specimen including isochromosome 8 formation and trisomy 5 and 9 (Figure 2C). Additionally, cytogenetic analysis of the SCCL-MT1 cell line demonstrates typical features of HNSCC and confirms the unique identity of this cell line (Figure 2B).

Phenotype of USC-HN2 cell line and heterotransplants closely resemble the original tumor biopsy

Immunophenotypic characterization of USC-HN2 cells in culture and tumors grown in Nude mice demonstrated similarity to the original tumor and confirmed a keratinizing squamous cell carcinoma (Figure 3). Neither the original tumor nor USC-HN2 cell line expressed

CD45, S100, or vimentin, consistent with its epithelial origin. USC-HN2 cells demonstrate positive expression of keratin, FABP5, E-cadherin, and CD44, as well as strong nuclear Rb and p53 expression *in situ*, consistent with HNSCC and the original tumor biopsy^{1,5,6,8,24}. EGFR and CD44 staining was increased in the cytospin and heterotransplant samples in comparison with the original tumor biopsy.

Flow cytometry studies were completed to characterize the phenotype of USC-HN2 compared with SCCL-MT1 (Table 1). Compared to isotype controls, both cell lines displayed positive staining for HNSCC biomarkers EGFR, CD24, E-cadherin, and CD44v6, whereas staining for CD74, CD133, and IL-13R α 2 was negative^{4,8,14,15}. Expression of stem cell-associated transcription factors c-KIT, NANOG, OCT3/4, and SOX2 was measured, and with the exception of positive staining for c-KIT in SCCL-MT1, these factors were not detected (data not shown)^{25,26}.

USC-HN2 has increased expression of immune modulatory cytokines

The expression of pertinent oncogenes and cytokines was examined for USC-HN2 and SCCL-MT1 using qRT-PCR techniques. USC-HN2 showed a statistically significant increase in mean expression of immune modulatory cytokines IL-1 β , IL-6, and IL-8 as compared to human reference RNA (Figure 4A, $p < 0.0005$), which was confirmed at the protein level by ELISA techniques (Figure 4B, $p < 0.05$). Both cell lines demonstrated significant protein secretion of GM-CSF and VEGF, though mRNA expression was not significantly increased for these genes. USC-HN2 also had increased TNF α protein levels compared with SCCL-MT1. The overall expression profile of USC-HN2 is highly immune modulatory and closely resembles that of SCCL-MT1.

To elucidate further the functional implications of the cytokine studies, both cell lines were assessed for their ability to induce Treg and MDSC suppressor cell populations from healthy volunteer peripheral blood mononuclear cells after one-week co-culture using methods established in our laboratory^{22,23}. Suppressive function of tumor-educated CD33⁺ MDSC or CD4⁺CD25^{high} Treg cells was assessed by their ability to inhibit the proliferation of fresh, autologous T cells stimulated with CD3/CD28 beads *in vitro*. USC-HN2 and SCCL-MT1 both induced strongly suppressive MDSC (Figure 4C) and weakly suppressive Treg cells (data not shown), consistent with previous reports that demonstrate HNSCC to be highly immune modulatory in patients^{7,22–24}.

Microarray gene expression analysis

Results of microarray gene expression analyses from USC-HN2 and SCCL-MT1 cell lines were compared with the data obtained from previously reported HNSCC tumor biopsy samples⁵. A total of 243 genes were significantly differentially expressed in both USC-HN2 and SCCL-MT1 cell lines. Many of the up-regulated genes identified were also present in HNSCC tumor biopsies, suggesting that USC-HN2 has an expression profile typical of HNSCC (Table 2).

Viral Screen and TP53 mutation analysis

Both cell lines, as well as the original tumor tissue used to derive USC-HN2 (SCCL-MT1 original tumor not available) were screened for HPV by *in situ* hybridization (Figure 2D). Consistent with the oral cavity origin of these cell lines, no evidence of HPV 16 or 18 was found^{3,21}. DNA from each of the cell lines was also screened for TP53 mutations, which are found in approximately half of all HNSCC tumors and are typically absent in HPV⁺ samples^{1,21}. TP53 mutations were identified in SCCL-MT1, but not in USC-HN2 (data not shown).

DISCUSSION

In this report, we describe the establishment and characterization of USC-HN2, a novel cell line derived from a patient with recurrent, invasive HPV⁻ buccal SCC with a past medical history significant for a 50-pack-year history of tobacco smoking and no pre-operative chemotherapy or radiation therapy. USC-HN2 cultured cells and heterotransplanted tumors closely resembled the original tumor biopsy specimen with respect to morphology, HNSCC-associated markers (keratin, E-cadherin, FABP5), HPV infection, and cytogenetic abnormalities. One difference noted was the outgrowth of a highly proliferative, EGFR⁺ subclone from a largely EGFR⁻ original tumor during establishment of the cell line. Overall, USC-HN2 showed similar morphology, growth rate, phenotype, and tumor suppressor and oncogene expression to the previously established HNSCC cell line SCCL-MT1.

Immune evasion and suppression are two mechanisms by which tumors escape immune destruction and evidence exists for the employment of both by HNSCC tumors^{10,11}. The results of this study revealed USC-HN2 and SCCL-MT1 to be highly immunogenic tumor models with strong immune suppression capacity. Additionally, the USC-HN2 cultured cells and heterotransplants, as well as the SCCL-MT1 cells, showed strong positivity for the cancer stem cell marker CD44v6. Cancer stem cell populations within tumors are reported to have greater expression of immunogenic tumor-associated antigens^{27,28}, a hypothesis that was supported here by microarray data demonstrating significant up-regulation of antigen-presentation-related genes in USC-HN2 and SCCL-MT1. In order for immunogenic tumor cells to persist in the face of infiltrating host immune cells, they must adapt to acquire immunosuppressive capabilities, such as the release of immune-inhibitory factors or the recruitment of immune suppressor cells¹¹. In this study we demonstrate that both USC-HN2 and SCCL-MT1 have strong immunosuppressive capabilities, including elevated expression of inflammatory and Th2 cytokines IL-1 β , IL-6, IL-8, GM-CSF, and VEGF. Previously, we have identified IL-1 β , IL-6, and GM-CSF as key factors for the induction of myeloid-derived suppressor cells, a population of innate immune suppressor cells that mediate direct suppression of effector T cells and expand regulatory T cell populations²². Indeed, co-culture of USC-HN2 and SCCL-MT1 with normal healthy donor PBMC generated functionally suppressive MDSC and Treg *in vitro*. Of note, when compared to six other established HNSCC cell lines (SCC-4, FaDu, Cal27, SW2224, Sw451, RPMI 2650) USC-HN2 and SCCL-MT1 were found to be the most potent inducers of suppressive MDSC, a finding which correlated with their high expression of immune modulatory cytokines²³.

Immunotherapy seeks to overcome tumor-mediated immune dysfunction and activate a cell-mediated immune response against cancer cells. Such an approach holds great promise for reducing damage to collateral tissue by taking advantage of the inherent specificity of the human immune system. Systemic trafficking and monitoring by immune cells also provides for superior treatment of metastatic and inoperable lesions compared with external beam irradiation and surgical therapies. Perhaps most importantly, the generation of immunologic memory following a robust anti-tumor immune response prevents the recurrence of tumors. While immune stimulatory treatment strategies have shown success in a variety of solid tumors, immunotherapeutic approaches in HNSCC have proven difficult perhaps in part due to the profound immune suppression generated by these tumors¹¹. New pre-clinical models are needed with which to study the mechanisms of immune suppression in HNSCC and develop new targeted immunotherapies. USC-HN2 and SCCL-MT1 appear to model highly immunogenic cancers with robust cytokine production and strong induction of suppressor cell populations as compared with other available HNSCC cell lines. Based upon these results, USC-HN2 and SCCL-MT1 provide excellent models for the development of new suppressor cell-targeted therapies for these difficult to treat tumors.

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Figure 1. Histology and morphologic analysis of USC-HN2

(A) (Left panels) H&E stained sections of the original tumor show groups of cells infiltrating the stroma with a desmoplastic and dense lymphoplasmacytic reaction, and occasional keratin pearl formation (arrow). Cells show increased nuclear to cytoplasmic ratio with prominent nucleoli and scattered mitotic figures (H&E x200 and x400 original magnification). (Right panels) Subcutaneous heterotransplantation of USC-HN2 cell line demonstrates a keratinizing tumor (arrow) that recapitulates the original tumor histology (H&E x200 and x400 original magnification). (B) Phase-contrast photomicrographs (top, x100 original magnification) and Wright-Giemsa-stained cytopins (bottom, x200 original magnification) of USC-HN2 and SCCL-MT1 cells. Both cell lines demonstrate squamous cell morphology with varied numbers of mitotic cells (rounded, luminescent cells).

Figure 2. Cytogenetic analysis and HPV Viral Screen of USC-HN2 and SCCL-MT1

(A) The karyotype of USC-HN2 shows a hyperdiploid cell line characterized by unbalanced translocation suspected to occur between the short arm of chromosome 2 and the distal long arm of chromosome 18, trisomy 5 and 9, partially trisomy for distal 2p, and tetrasomy for 8q with a modal number of 50 chromosomes. (B) The karyotype of SCCL-MT1 also shows a hypertriploid cell line with characteristic features of HNSCC including multiple deletions, isochromosome formation, and breakpoints at or near the centromeres. (C) Multi-color FISH to verify that the USC-HN2 cell line was derived from malignant cells present in the primary tumor. Cell line signal patterns correlated very well with the original tumor. (D) Single color FISH using an HPV16/18 probe demonstrates the HPV⁻ status of USC-HN2 and SCCL-MT1 cell lines as compared with the HPV⁺ control cell line HeLa.

Figure 3. Characterization of the original tumor biopsy, USC-HN2 cell line, and heterotransplanted tumor

Photomicrograph of immunoperoxidase staining of original tumor biopsy (left panels), USC-HN2 cells from culture in cytopsin preparations (middle panels), and formalin-fixed paraffin-embedded tissue sections of USC-HN2 Nude mouse subcutaneous heterotransplant (right panels) for CD45, S100, Vimentin, p53, Rb, EGFR, FABP5, E-cadherin, CD44, and Keratin (x400 original magnification).

Figure 4. USC-HN2 is highly immunomodulatory and induces suppressor cells

(A) qRT-PCR analysis of cytokine mRNA levels in USC-HN2 and SCCL-MT1 compared with human reference RNA. Both cell lines both showed increased expression of IL-1 β , IL-6, IL-8, and COX2. (B) Secreted protein levels measured by ELISA confirmed similar, highly immunomodulatory cytokine profiles for USC-HN2 and SCCL-MT1. (C) USC-HN2 and SCCL-MT1 induced strongly suppressive MDSC after one-week co-culture with healthy donor PBMC. For all samples mean (n $\geq$ 2) data shown +SD; *indicates p<0.05.

Table 1
Analysis of USC-HN2 surface markers by FACS

Flow cytometry studies of USC-HN2 and SCCL-MT1 demonstrate surface markers characteristic of HNSCC cell lines. Percent of positive staining cells (middle columns) and mean fluorescence intensity (MFI, right columns) are shown for each antibody target and isotype control. Positive findings are shown in bold.

Target	% Positive		MFI	
	Isotype Control	Antibody	Isotype Control	Antibody
<u>USC-HN2</u>				
CD24	0.90	76.11	56.76	609.77**
E-cadherin	0.90	35.81	56.76	303.60**
EGFR	0.72	92.84	21.38	479.34**
CD44v6	0.90	7.75	56.76	152.86*
CD74	0.90	0.49	56.76	41.59
CD133	0.79	0.61	32.68	26.84
IL-13R32	0.38	0.24	19.23	12.15
<u>SCCL-MT1</u>				
CD24	1.37	24.7	65.13	203.06**
E-cadherin	1.37	8.87	65.13	215.69**
EGFR	0.34	98.34	16.20	1392.73**
CD44v6	1.37	6.03	65.13	133.36*
CD74	1.37	0.61	65.13	49.12
CD133	1.32	0.98	31.02	27.16
IL-13R32	1.04	0.27	24.13	13.44

* MFI 50–100 above isotype control

** MFI >100 above isotype control

Table 2
Selected up-regulated genes identified in USC-HN2 and SCCL-MT1 cell lines also present in HNSCC tumor biopsies

Log₂ ratio of 1 signifies a 2-fold difference in the mean gene expression of the cell line versus human reference RNA ($p < 0.05$).

GeneBank Access ID	Gene Symbol (Annotation)	Log ₂ Ratio
<i>Immune Response</i>		
NM_002117	HLA-C (major histocompatibility complex, class I C)	2.6
NM_004048	B2M (beta-2 microglobulin)	2.1
NM_005514	HLA-B (major histocompatibility complex, class I B)	1.8
NM_002116	HLA-A (major histocompatibility complex, class I A)	1.7
NM_013230	CD24 (CD24 antigen)	1.3
<i>Cell Growth, Maintenance/Cell cycle Regulation</i>		
NM_000424	KRT5 (keratin 5)	2.9
NM_000526	KRT14 (keratin 14)	2.0
NM_033666	ITGB1 (integrin, beta 1)	2.0
NM_002273	KRT8 (keratin 8)	1.5
NM_006088	TUBB2C (tubulin beta 2C)	1.5
NM_006082	TUBA1B (tubulin alpha 1b)	1.4
NM_005507	CFL1 (cofilin 1)	1.3
NM_002628	PFN2 (profilin 2)	1.3
NM_005022	PFN1 (profilin 1)	1.0
NM_004360	CDH1 (E-cadherin)	1.0
<i>Translation and Protein Synthesis</i>		
NM_000971	RPL7 (ribosomal protein L7)	1.7
NM_006013	RPL10 (ribosomal protein L10)	1.4
NM_000979	RPL18 (ribosomal protein L18)	1.2
NM_001042559	EIF4G2 (translation initiation factor 4 gamma 2)	1.2
NM_001006	RPS 3A (ribosomal protein S3A)	1.2
<i>Metabolism</i>		
NM_001135700	YWHAZ (tyrosine-3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta)	2.5
NM_002808	PSMD2 (proteasome 26S subunit)	1.8
NM_002794	PSMB2 (proteasome subunit beta 2)	1.6
NM_021130	PPIA (peptidylprolyl isomerase A (cyclophilin A))	1.5
NM_005561	LAMP1 (lysosomal-associated membrane protein 1)	1.4
NM_001165415	LDHA (lactate dehydrogenase A)	1.4
NM_005348	HSP90AA1 (heat shock 90kDa alpha class A member 1)	1.4
NM_001689	ATP5G3 (ATP synthase H ⁺ transporting subunit)	1.0
NM_002715	PPP2CA (protein phosphatase 2 catalytic subunit)	1.0
<i>Others</i>		
NM_005978	S100A2 (S100 calcium binding protein A2)	2.8
NM_005953	MT2A (metallothionein 2A)	2.6
NM_003329	TXN (thioredoxin)	2.3

GeneBank Access ID	Gene Symbol (Annotation)	Log ₂ Ratio
NM_006096	NDRG1 (N-myc downstream regulated 1)	2.2
NM_021103	TMSB10 (thymosin, beta 10)	1.9
NM_021009	UBC (ubiquitin C)	1.7
NM_199185	NPM1 (nucleophosmin)	1.6
NM_001428	ENO1 (enolase 1)	1.2